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Optimum separation condition of peptides in reversed-phase liquid chromatography

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Abstract

An efficient optimization method was suggested to separate biologically active peptides by RP-HPLC. In this work, the binary mobile phase of water and acetonitrile was used with the buffer of trifluoroacetic acid (TFA). The elution profiles were calculated by the plate theory based on the linear and quadratic equations of retention factor, $\ln k = A + BF$, $\ln k = A + BF + CF^2$, and F was the vol.% of acetonitrile. We modified the plate theory to calculate elution profile in both isocratic and gradient mode. From the final calculated results, the first mobile phase composition was water in 0.1% TFA/acetonitrile in 0.1% TFA, 81/19 vol.%, then after 7–8 min, the second composition of mobile phase was linearly changed to 79/21 vol.%, and finally after 8 min, it was kept at the isocratic mode. In the experimental conditions, the agreement between the experimental data and the calculated values was relatively good.

Keywords: Mobile phase composition; Plate theory; Peptides

1. Introduction

Optimization in reversed-phase high performance liquid chromatography (RP-HPLC) involves the selection of experimental condition for adequate separation and acceptable retention time for each individually samples. But, in chemical and pharmaceutical laboratories obtaining a balance between resolution and analysis time is not always easy. An efficient optimization method should be employed during the method development process in order to deal with these optimization problems. Computers have been used as an aid in high performance liquid chromatography method development since the late 1970s [1–3]. The method developments by software were frequently demonstrated, and the applications using the software have been increasing [4–8]. Especially the optimization scheme was designed and programmed, so the resulting HCI software was developed by High-Purity Separation Lab., Inha University, for the purpose of the optimization of chromatographic separation. The scope of HCI program was limited to analytical condition. It could be utilized in normal-phase as well as reversed-phase liquid chromatography for both the isocratic and gradient modes. The basic function enabled to predict the retention time of samples in a given mobile phase composition, and additionally column efficiency and resolution, elution profile of sample in specific and optimized operating conditions.

In this work, the optimization of mobile phase condition for four biological-active peptides was determined by the software of HCI program. Naturally occurring peptides show a wide variety of biological effects. The main function of bradykinin is to increase the sensation of pain [9]. Bradykinin also sensitizes free nerve endings, making them hypersensitive to heat and light touch and creating an overall sensation of soreness. Leucine enkephalins have been associated with addiction to and withdrawal from morphine [10]. This decrease in enkephalin neural activity necessitates an increase in the amount of morphine in order to maintain the same level of analgesia. Its presence in the blood launches a chain of reactions that result in the production of angiotensins II and III [11], a molecule that raises blood pressure. In addition to its role in raising blood pressure, angiotensin II promotes the overgrowth of cells, called hypertrophy, in the heart and blood vessel walls and in the kidneys. The hypertrophic response to angiotensin II is a major problem leading to heart failure, atherosclero-

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sis, and kidney failure. RP-HPLC is the most widely used analytical technique for separation and isolation of such as the peptides [12,13]. So, the mobile phase is a mixture of an aqueous and organic solvent in which the hydrophobic interaction between peptides and the non-polar stationary phase allows peptide isolation [14,15].

HCI program was used to optimize the separation of the four peptides by RP-HPLC. A modified equation was suggested for calculating the distances migrated by the solutes in step and gradient mode. The optimum composition of mobile phase for the separation of the four peptides was obtained on the basis of resolutions and separation times. The elution profiles in the optimal mobile phase condition and operating mode were calculated by plate theory to compare with experimental data.

2. Theoretical background

In this work, the logarithmic retention factor, k, is correlated by a linear relationship and quadratic relationship involving the vol.% (F) of organic modifier [16,17].

$$\ln k = A + BF \tag{1}$$

$$\ln k = A + BF + CF^2 \tag{2}$$

where A, B, and C are empirical constants which should be experimentally determined. Eqs. (1) and (2) were applied to binary mobile phase in reverse-phase HPLC.

Retention volume in isocratic mode is expressed by retention factor as follows:

$$V_{r,n} = V_0(1 + k_n) \tag{3}$$

where $V_{r,n}$, and k_n are the retention volume and retention factor in the nth mobile phase composition respectively, and V_0 is the dead volume of unretained compound. The prediction of retention time under gradient conditions has been described by assuming that a gradient step is similar to a sequence of short isocratic steps [18,19]. The modified equation is proposed for predicting the retention volume of step-gradient elution:

$$V_{Rg} = V_0(1 + k_2) + \frac{V_{g,1}}{k_1}(k_1 - k_2) \tag{4}$$

where k_1 and k_2 are the retention factors in the first and second mobile phase compositions, and obtained by Eqs. (1) and (2). $V_{g,1}$ is the volume of the first mobile phase in the step-gradient elution passing through an inlet of the chromatographic column until the second mobile phase is introduced to the column inlet [20]. It can be calculated by summation of a mixer volume installed in HPLC and gradient volume. In the case of linear-gradient mode, mobile phase composition keeps changing gradually and continuously. As the linear-gradient mode may be envisaged as the infinite small segments of step gradient, Eq. (4) is modified and extended to linear-gradient mode. The retention volume in the linear-gradient mode can be calculated:

$$V_{Rg} = V_{r,\infty} + (V_{r,\infty} - V_0)\sum_{i=1}^{\infty}\frac{V_{g,i}(V_{r,i} - V_{r,i+1})}{(V_{r,i} - V_0)^2} \tag{5}$$

The number of theoretical plates, N, is calculated in isocratic mode,

$$N = 16\left(\frac{t_R}{w}\right)^2 \tag{6}$$

The number of theoretical plates is assumed to be independent of the mobile phase composition throughout this work. It was obtained by the average value from several runs. In gradient mode, the number of theoretical plate was calculated by substituting t_R into t_{Rg} in Eq. (6), w was calculated by Eq. (6) substituting t_R into t_{Rg} or $t_{R,n}$ according to mobile phase shape and inserting average value of N. The resolution between component 1 and 2 is given by:

$$R_{12} = \frac{2(t_{R_1} - t_{R_2})}{w_1 + w_2} \tag{7}$$

The optimum resolution was obtained by calculating the retention time and peak width from Eqs. (4)–(6). According to the plate theory, the chromatographic column is mathematically equivalent to a plate column where the total length is divided into N. It is assumed that instantaneous equilibrium is established for the solute between mobile and stationary phases. A material balance on solute around the plate N leads to the following equation [18,20]:

$$c_N = c_0\sum_{i=N-r}^{N-1}\frac{(aV)^i}{i!}e^{-aV} \tag{8}$$

where c_N is the outlet concentration of solute, c_0 initial concentration, and a a constant,

$$a = \frac{1}{v_m + Kv_s} \tag{9}$$

where v_m and v_s are the volume of mobile and stationary phases in a theoretical plate, respectively. Eq. (8) enables to predict concentration elution profile of each component. The equilibrium constant (K) is correlated in terms of partition coefficient as:

$$K = \left(\frac{\varepsilon}{1 - \varepsilon}\right)k \tag{10}$$

where ε is the total porosity of the chromatographic column, and it is assumed 0.75.

3. Experiments

3.1. Reagents

Four standard peptides, Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), [Val^4]-Angiotensin III (Arg-Val-Try-Val-

His-Pro-Phe), Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and [D-Ala^2]-Leucine enkephalin (Tyr-D-Ala-Gly-Phe-Leu), were purchased from Sigma (St. Louis, MO, USA). HPLC grade solvent, acetonitrile was from Ducksan Pure Chemical (Kyungki-Do, Korea). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO, USA). Water filtered by Milipore ultra pure water system (Milipore, Bedford, MA, USA).

3.2. Sample preparation

Four standard peptides, 5 mg, were dissolved in water 1 ml, then the concentration of the solutions were adjusted to 5000 μg/ml, respectively. The constant injection volume of mixtures solution, 3 μl, was used throughout.

3.3. Apparatus and method

HPLC was performed using Waters 600S solvent delivery system (Waters, Milford, MA, USA). The 2487 UV dual channel detector was used (Waters, Milford, MA, USA). Data acquisition system was Millenium^{32} (Waters) installed

Table 2
Empirical constants and regression coefficients of peptides by a linear relationship

Material	$\ln k = A + BF$		Regression coefficient
	A	B	
Angiotensin III	3.974	−0.137	0.9855
Leucine enkephalin	3.840	−0.119	0.9842
Bradykinin	4.955	−0.164	0.9829
Angiotensin II	5.091	−0.161	0.9772

Table 3
Empirical constants and regression coefficients of peptides by a quadratic relationship

Material	$\ln k = A + BF + CF^2$			Regression coefficient
	A	B	C	
Angiotensin III	5.829	−0.279	0.0025	0.9994
Leucine enkephalin	5.522	−0.248	0.0023	0.9993
Bradykinin	7.342	−0.348	0.0032	0.9987
Angiotensin II	7.840	−0.372	0.0037	0.9989

Table 1
Retention factors of peptides in binary mobile phases

Mobile phase (vol.%)		Retention factors (k)			
Water + 0.1% TFA	Acetonitrile + 0.1% TFA	Angiotensin III	Leucine enkephalin	Bradykinin	Angiotensin II
60	40	0.38	0.59	0.35	0.44
65	35	0.55	0.87	0.59	0.70
70	30	0.91	1.33	1.00	1.23
75	25	1.68	2.29	2.10	2.50
80	20	4.10	4.83	5.85	7.01
85	15	6.57	7.93	12.1	15.1

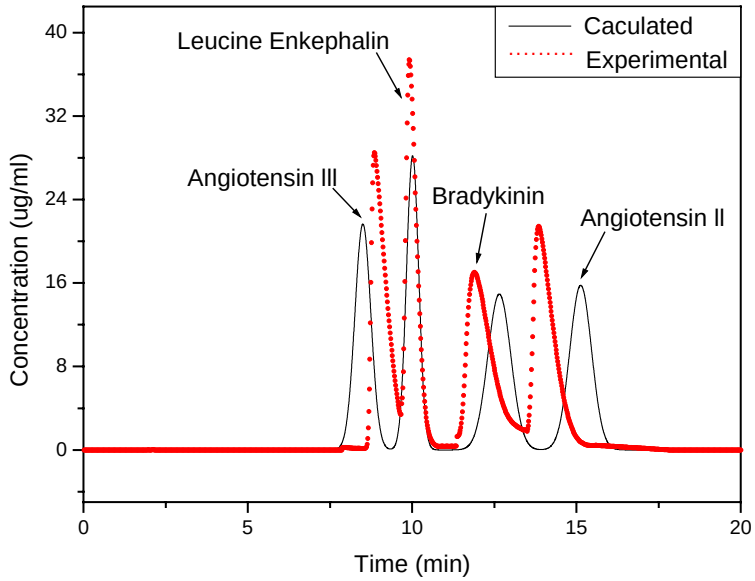

Fig. 1. Comparison of the experimental and calculated profiles by Eq. (1) in isocratic condition (F: 19%).

in HP Vectra 500 PC. The mobile phases were degassed with helium. The flow rate of mobile phase was 1 ml/min and monitored at the fixed wavelength of 215 nm. The column was purchased from Alltech Co. The column size was 0.46 cm × 15 cm and packed by C18, 100 Å, 5 μm. All the experimental runs were carried out in ambient temperature. The dead volume (V_0) was determined as the retention volume of 20 μl of acetonitrile.

4. Results and discussion

To investigate the relationship of retention factor in terms of composition in mobile phase, several experimental runs were performed on an isocratic mode. Besides, the required input data for HCI program included the number of theoretical plates, dead volume of the column, specification of a column (diameter and length), diameter of packings, and flow rate of mobile phase to predict elution profiles through a chromatographic column. The retention times of four peptides were measured with different contents of organic modifier. If the retention factor is expressed as a function of mobile phase composition, the elution profile of a solute might be estimated for any change in mobile phase composition by HCI program. The experimental data of retention factor (k) of samples and vol.% of organic modifier (F) were listed in Table 1. The linear correlation between $\ln k$ and mobile phase composition was assumed as in Eq. (1),

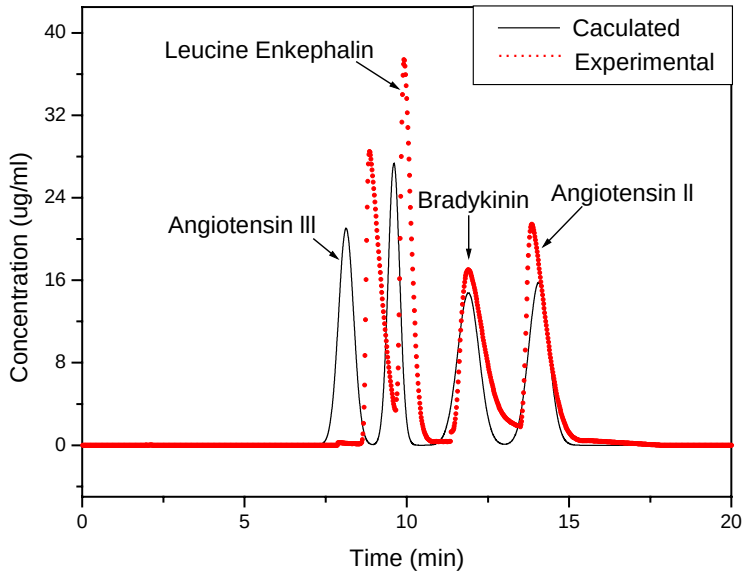

Fig. 2. Comparison of the experimental and calculated profiles by Eq. (2) in isocratic condition (F: 19%).

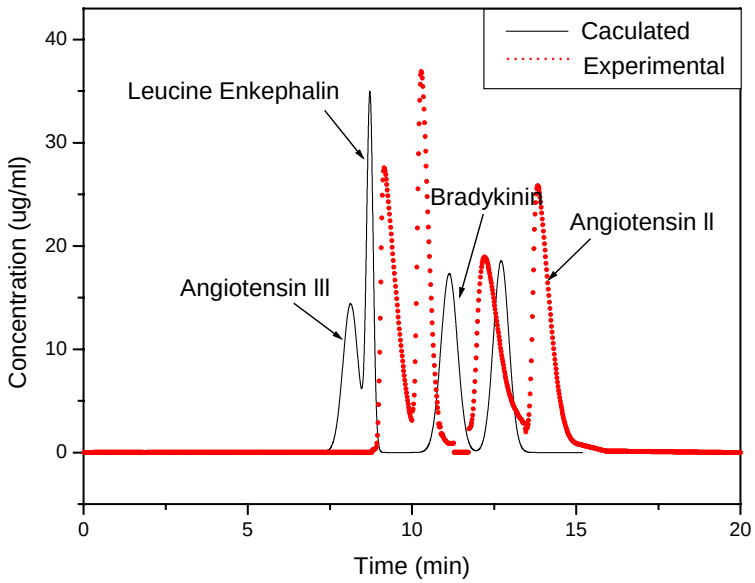

Fig. 3. Comparison of the experimental and calculated profiles by Eq. (1) in gradient condition (F_1: 19%, F_2: 21%, $V_{g,1}$: 7 min, $V_{g,\infty}$: 8 min).

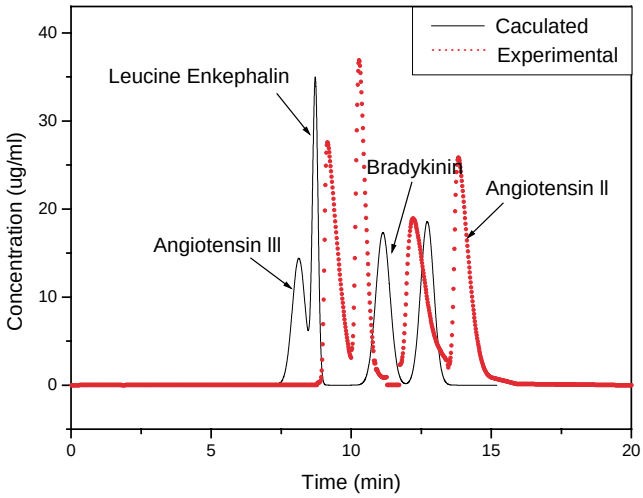

Fig. 4. Comparison of the experimental and calculated profiles by Eq. (2) in gradient condition (F_1: 19%, F_2: 21%, $V_{g,1}$: 7 min, $V_{g,\infty}$: 8 min).

while the quadratic equation as in Eq. (2). Their regression analyses were presented in Tables 2 and 3, respectively. The regression coefficients of four peptides were almost to 1.00 in Eq. (2).

On isocratic mode, the binary system of 0.1% TFA in water and 0.1% TFA in acetonitrile was used. The elution order of four peptides were Angiotensin III, Leucine enkephalin, Bradykinin and Angiotensin II, and it was not changed with mobile phase composition. In Fig. 1, the experimental data were observed at the mobile phase composition of 0.1% TFA in water/0.1% TFA in acetonitrile, 81/19 vol.%, and the calculated values were obtained by HCI program with Eq. (1). Contrary to Fig. 1, the calculated values in Fig. 2 with Eq. (2). The experimental and calculated profiles were in better agreements with Fig. 1 more than Fig. 2.

On gradient mode, the first mobile phase composition was water in 0.1% TFA/acetonitrile in 0.1% TFA, 81/19 vol.%, then after 7 min to 8 min, the second composition of mobile phase was linearly changed to 79/21 vol.%, and finally after 8 min, it was kept at the isocratic mode. Similar to Figs. 1 and 2, the calculated values by Eqs. (1) and (2) were compared with experimental data from gradient mode in Figs. 3 and 4, respectively. The agreement between the experimental data and calculated values of the Bradykinin, and Angiotensin II in Fig. 4 was relatively good. On the isocratic condition, the linear equation of Eq. (1) was sufficient to estimate the calculated profiles, as shown in Fig. 1. However, on the gradient condition, the quadratic equation of Eq. (2) was better to fit the experimental data. Some deviation from experimental data might be attributed to the curvature of the gradient (gradient dispersion or mixing) at the junction of a segmented gradient.

Comparisons of the calculated resolutions by Eqs. (1) and (2) in both isocratic and gradient modes with the experimental variables were summarized in Table 4 and Fig. 5.

Table 4
Comparison of resolutions and elution methods between experimental data and calculated values

Resolution[a]	$\ln k = A + BF$					$\ln k = A + BF + CF^2$					
	Isocratic		Error[b] (%)	Gradient		Error (%)	Isocratic		Error (%)	Gradient		Error (%)
	Experimental	Calculated		Experimental	Calculated		Experimental	Calculated		Experimental	Calculated	
R_1	1.01	1.50	32.7	1.04	0.76	26.9	1.01	1.53	34.0	1.04	2.36	55.9
R_2	1.62	2.10	22.9	1.59	2.95	46.1	1.62	1.92	15.6	1.59	1.74	8.6
R_3	1.31	1.54	14.9	1.19	1.39	14.4	1.31	1.44	9.0	1.19	1.41	15.6

[a] R_1: Angiotensin III and Leucine enkephalin, R_2: Leucine enkephalin and Bradykinin, R_3: Bradykinin and Angiotensin II.
[b] $(R_{exp}-R_{cal})/(R_{exp}) \times 100$.

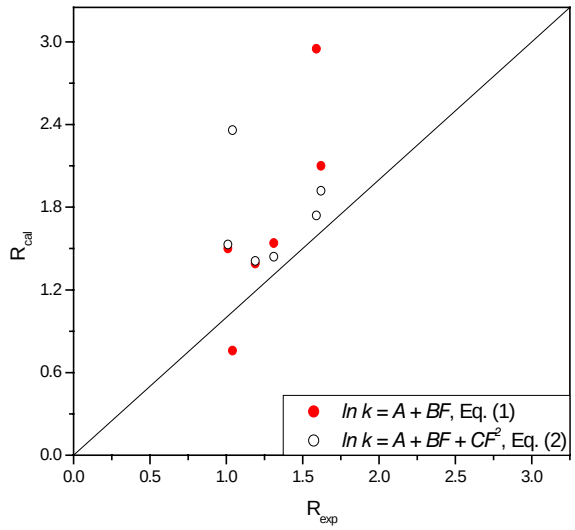

Fig. 5. Comparisons of the experimental and calculated resolutions by Eqs. (1) and (2).

5. Conclusion

The four peptides were separated by change in mobile phase compositions. Based on the plate theory, elution profiles were predicted by introducing the concept of solute migration in mobile phase with the linear and the quadratic dependency of $\ln k$ in terms of the content of organic modifier. With the aid of HCI program, the recommended experimental conditions of mobile phase composition and gradient step were suggested, and the calculated elution profiles by the quadratic relationship of $\ln k$ showed better coincidence with the experimental data than linear correlation.

6. Nomenclature

A, B, C	empirical constants used in Eqs. (1) and (2)
c_0	concentration of injected solute (mg/ml)
c_N	concentration of solute in the Nth plate (mg/ml)
F, F_1, F_2	vol.% of organic modifier in mobile phase, in the first, and second mobile phase, respectively
K	equilibrium constant
k	retention factor
N	number of theoretical plates
t_R	retention time (min)
V	volume of mobile phase passing through the column (ml)
$V_{c,n}$	volume of mobile phase at solute migration meet with mobile phase migration in the nth mobile phase (ml)
$V_{g,I}$	volume of the ith mobile phase passing through a column inlet before the introduction of the $(i+1)$th mobile phase into the column
V_0	dead volume (ml)
V_{Rg}	retention volume (ml)
$V_{r,1}$, $V_{r,\infty}$	retention volume in the initial and final mobile phase, respectively (ml)
v_m	volume of mobile phase in a theoretical plate (ml)
v_s	volume of the stationary phase in a theoretical plate (ml)
w	band width in the baseline (min)

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